Increasing Epidermal Growth Factor Receptor Expression in Human Melanocytic Tumor Progression

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Different results have been reported on the expression of epidermal growth factor receptor (EGFR) in human melanocytic lesions, which may be due to different methodologic approaches. Therefore, we compared EGFR expression in six human melanoma cell lines by utilizing the monoclonal antibodies 2E9, 425, and 225, applying four immunocytochemical staining procedures. The results were compared with those obtained by a multiple point ligand binding assay. In addition, Northern blot analysis was performed.

A three-step immunoperoxidase method using the monoclonal antibody 2E9 proved most sensitive. Staining intensities, estimated semiquantitatively, correlated well with the quantitative data obtained by the ligand-binding assay. Expression on the mRNA level was also in agreement with these results.

Immunohistochemical staining of a large series of human cutaneous melanocytic lesions using the method selected showed differential EGFR expression in various stages of melanocytic tumor progression: 19% of common nevocellular nevi; 61% of dysplastic nevi, 89% of primary cutaneous melanomas, and 91% of melanoma metastases showed staining of the melanocytic cells. Intralesional heterogeneity of EGFR expression was present. Although the mean percentage of positive melanocytic cells in positive lesions did not increase with progression, mean staining intensity was stronger in malignant lesions compared to benign lesions.

Ligand binding assays showed that EGFR expression in the highly metastasizing cell lines MV3 and BLM was at least 40 times higher than in the cell lines IF6, 530, M14, and Mel57, which do not or only sporadically metastasize after subcutaneous inoculation in nude mice. Although the differences between the various stages of progression are not absolute, we provide further evidence that EGFR expression increases in human melanocytic tumor progression. *J Invest Dermatol 99:168–173, 1992*

Antigens associated with tumor progression of human melanocytic cells have been identified and are detectable immunohistochemically [1–7]. Different results have been reported on the expression in human melanocytic lesions of one of these antigens, the epidermal growth factor receptor (EGFR) [7,8].

The EGFR is a 170-kD transmembrane glycoprotein [9]. The cytoplasmatic domain of the receptor is in structure similar to the v-erbB oncogene protein [10]. Binding of epidermal growth factor (EGF) or transforming growth factor α to the external domain of EGFR can result in the activation of a mitogenic pathway [11]. In vitro, overexpression of the human EGFR confers an EGF-dependent transformed phenotype to NIH 3T3 cells [12].

Elder et al [7] reported that the EGFR shows the distribution of a progression antigen in human melanocytic lesions. However, in another series of human melanocytic lesions studied immunohistochemically by Real et al [8] no EGFR expression was found. As these authors used different immunohistologic techniques and different monoclonal antibodies (MoAb), we wondered whether this could explain the dissimilar results. Therefore, we first compared the sensitivity of four routinely applied immunohistochemical methods using a panel of six human melanoma cell lines with different levels of EGFR expression. The MoAb used by Elder et al (MoAb 425) [13] and by Real et al (MoAb 225) [14] were compared with a third one, MoAb 2E9 [15], which appeared very sensitive in a pilot study. In addition, the level of EGFR expression in the cell lines was quantified using a multiple-point ligand-binding assay. Furthermore, EGFR-mRNA expression was determined. Based on these results the most sensitive immunohistochemical method was chosen for further study of EGFR expression in a large series of melanocytic lesions. We provide further evidence for a differential expression of EGFR, both in human lesions of various stages of melanocytic tumor progression and in human melanoma cell lines with a different metastatic behavior in nude mice.

MATERIALS AND METHODS

Cell Lines Human melanoma cell lines used included the following: IF6 [16], 530 [17], Mel 57 [18], M14 [19], BLM [16], and MV3

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Abbreviations:
ABC: avidine-biotine complex
DN: dysplastic nevus
EGFR: epidermal growth factor receptor(s)
(m)EGF: (mouse) epidermal growth factor
MM: melanoma metastasis
MoAB: monoclonal antibody(s)
ND: not determined
NN: nevocellular nevus
PAP: peroxidase–anti-peroxidase
PM: primary melanoma
RAMPO: peroxidase-labeled rabbit anti-mouse immunoglobulin

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[20]. Cell lines IF6, BLM, and MV3 were developed in our laboratory. BLM is a subline of the BRO line [21] with a higher metastatic potential in nude mice than the parental line. Furthermore, the vulval carcinoma cell line A431 was used [22]. All cell lines were grown as monolayers in culture flasks on Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, glutamine, penicillin G, and Streptomycin. Cells were harvested by short trypsinization and processed for cytospin preparation or RNA isolation.

Human Melanocytic Lesions Subcentral representative slices of 16 common nevocellular nevi (NN), 13 dysplastic nevi (DN), 48 primary cutaneous melanomas (PM), and 22 melanoma metastases (MM) were snap-frozen in liquid N_2 and stored at −70°C until use. The histopathologic diagnosis was made on corresponding paraffin sections and the representativity of the samples of the frozen sections was ascertained. Primary melanomas were divided into three categories according to the Breslow thickness [23]: ≤0.76 mm (PM1), between 0.76 and 3 mm (PM2), and ≥3 mm (PM3) in thickness. The degree of atypia in DN was assessed [24].

MoAb and Immunocytochemical Techniques MoAb included 1) 2E9 (ascites fluid) [15]; 2) 425 (tissue culture supernatant) [13]; 3) 225 (1 mg/ml purified immunoglobulins) [14]. All three murine MoAb recognize an antigenic determinant of the protein core of the EGFR. Cytospin preparations of the cell lines were dried, fixed in acetone, and incubated for 60 min with MoAb 2E9 (1:25), MoAb 425 (undiluted), or MoAb 225 (1:12.5). The further procedure consisted of one of four different immunoperoxidase techniques: 1) a two-step method, incubation with peroxidase-labeled rabbit anti-mouse Ig serum (RAMPO, Dakopatts, Denmark); 2) a three-step method, incubation with RAMPO followed by incubation with peroxidase-labeled swine anti-rabbit Ig serum (Dakopatts, Denmark); 3) a PAP method, incubation with rabbit anti-mouse Ig serum (Cappel, Belgium), followed by incubation with swine anti-rabbit Ig serum (Dakopatts, Denmark) and incubation with rabbit PAP complex (Dakopatts, Denmark); and 4) an avidin-biotin-complex (ABC) method (Vector Laboratories, USA). All incubations were performed at room temperature for 30–60 min. Between incubations sections were rinsed with PBS.

Visualization of the immunoperoxidase reactions was performed with 3-amino-9-ethylcarbazole as substrate. Preparations were counterstained with hematoxylin. Titration experiments with the MoAb were performed using method 2.

Based on the staining results of the cytospin preparations, air-dried and acetone-fixed frozen 4-μm sections of melanocytic lesions were processed using MoAb 2E9 and the three-step immunoperoxidase technique (method 2).

Score The intensity of staining in the melanocytic cells was scored semiquantitatively as −(none), ±(weak), +(moderate), or ++(marked).

In the cytospin preparations of the cell lines the percentages of positive cells and the average staining intensities were estimated. In the frozen sections of human melanocytic lesions the percentages of positive melanocytic cells were estimated. Lesions were regarded EGFR positive if 5% or more of the melanocytic cells in the lesion stained.

Ligand-Binding Assay Cells were harvested with a rubber policeman and homogenized by means of ultrasound bursts (MSE Soniprer-150: nominal frequency 23 kHz, amplitude 10 μm) for 10 seconds, on ice, in EGFR assay buffer (0.02 M phosphate buffer, pH 7.4, containing 0.15 M NaCl and 70 μg/ml Bacitracin).

The homogenates were centrifuged for 10 min at 800 × *g*, 4°C, to spin down nuclei and other coarse cell fragments. The supernatants were recentrifuged for 60 min at 105,000 × *g*, 4°C. The cell-membrane pellets thus obtained were resuspended in 1.1 ml of EGFR assay buffer by means of ultrasound bursts. EGFR assays were performed in a manner similar to that described previously [25]. To summarize: eight times 100 μl aliquots of cell-membrane preparation were incubated with ^{125}I-mouse-EGF (mEGF) tracer at concentrations ranging from 0.15 to 3.5 nM. Aspecific binding was assessed in duplicate using 1 nM ^{125}I-mEGF and a 250-times excess of unlabeled mEGF. Receptor-bound and free ligand were separated using hydroxyapatite. Receptor values were calculated by Scatchard analysis and expressed in fmol/mg of membrane protein.

Table I. Expression of EGFR Protein and EGFR mRNA in Human Melanoma Cell Lines and Cell Line A431

	A[a]							
	Two-Step Method			Three-Step Method[b]				
Cell Line	425	225	2E9	425	225	2E9	B[a]	C[a]
IF6	—	—	—	—	—	—	—[c]	—[d]
530	—	—	—	—	—	±	10.5	±
M14	—	—	—	—	—	±	33.9	±
Mel 57	—	—	—	—	—	—	—[c]	—[d]
BLM	—	—	+	+	+	++	1436	++
MV3	—	—	+	+	+	++	1480	++
A431	ND	ND	ND	ND	ND	+++	17271	+++

[a] A, EGFR expression as detected immunohistochemically using the monoclonal antibodies 425, 225, and 2E9. B, EGFR expression in fmol per mg of cell membrane protein as determined with a ligand binding assay. C, EGFR mRNA expression determined by Northern blot analysis.
[b] Same scores were obtained using the three step, the PAP, or the ABC method.
[c] —, not detectable (<6 fmol/mg).
[d] —, no signal; ±, faint signal; ++, marked signal; +++, strong signal.

Northern Blot Analysis Total RNA of the cell lines was isolated by the LiCl/urea method [26]. Ten-microgram aliquots of total RNA were glyoxylated [27] and size fractionated on a 1% agarose gel followed by blotting on hybond-N-membrane according to the procedure recommended (Amersham, UK).

Hybridizations were performed as described [28]. To detect EGFR mRNA the 0.8-Kb EcoRI fragment of Lambda HER A 64 was used as a probe [29]. For control hybridizations on the amount of RNA a 28S rRNA probe was used. Both probes were radiolabeled using the multiprime labeling method (Amersham, UK).

RESULTS

Immunocytochemistry of the Cell Lines Table I *(columns A)* summarizes data on the EGFR expression in the melanoma cell lines and cell line A431 as obtained by applying different MoAb and immunocytochemical staining techniques. For each separate MoAb, the intensities of the staining signals were similar when comparing the three-step immunoperoxidase technique, the PAP technique, and the ABC technique. The two-step technique was less sensitive. With regard to the MoAb used at the indicated dilutions, a less insensitive or negative staining result was obtained with MoAb 425 and MoAb 225, compared with MoAb 2E9. Titration experiments showed similar staining signals for the MoAb 2E9, 425, and 225, when used diluted 1:300, undiluted, and diluted 1:12.5, respectively. Using the MoAb 2E9 (1:25) and the three-step immunoperoxidase detection method, cell lines IF6 and Mel 57 were negative, 530 and M14 weakly positive, and BLM and MV3 markedly positive (Fig 1*A–C*). The positive reference cell line A431 showed a very strong staining. In the positive melanoma cell lines 80–100% of the tumor cells stained.

Ligand-Binding Assay of the Cell Lines In Table I *(lane B)* and Fig 2 of the level of EGFR expression in the cell lines, as quantified by radioligand-binding assays is shown. Cell line A431 showed a very high expression. Cell lines MV3 and BLM showed a high, cell lines M14 and 530 a low, and cell lines IF6 and Mel 57 no measurable level of expression. All the EGFR containing melanoma cell lines showed one single class of high-affinity EGFR binding sites. In contrast to this, the A431 cell line, containing high levels of EGFR, showed a curvilinear Scatchard-plot (linearized in Fig 2), indicating the presence of two EGFR binding sites with different

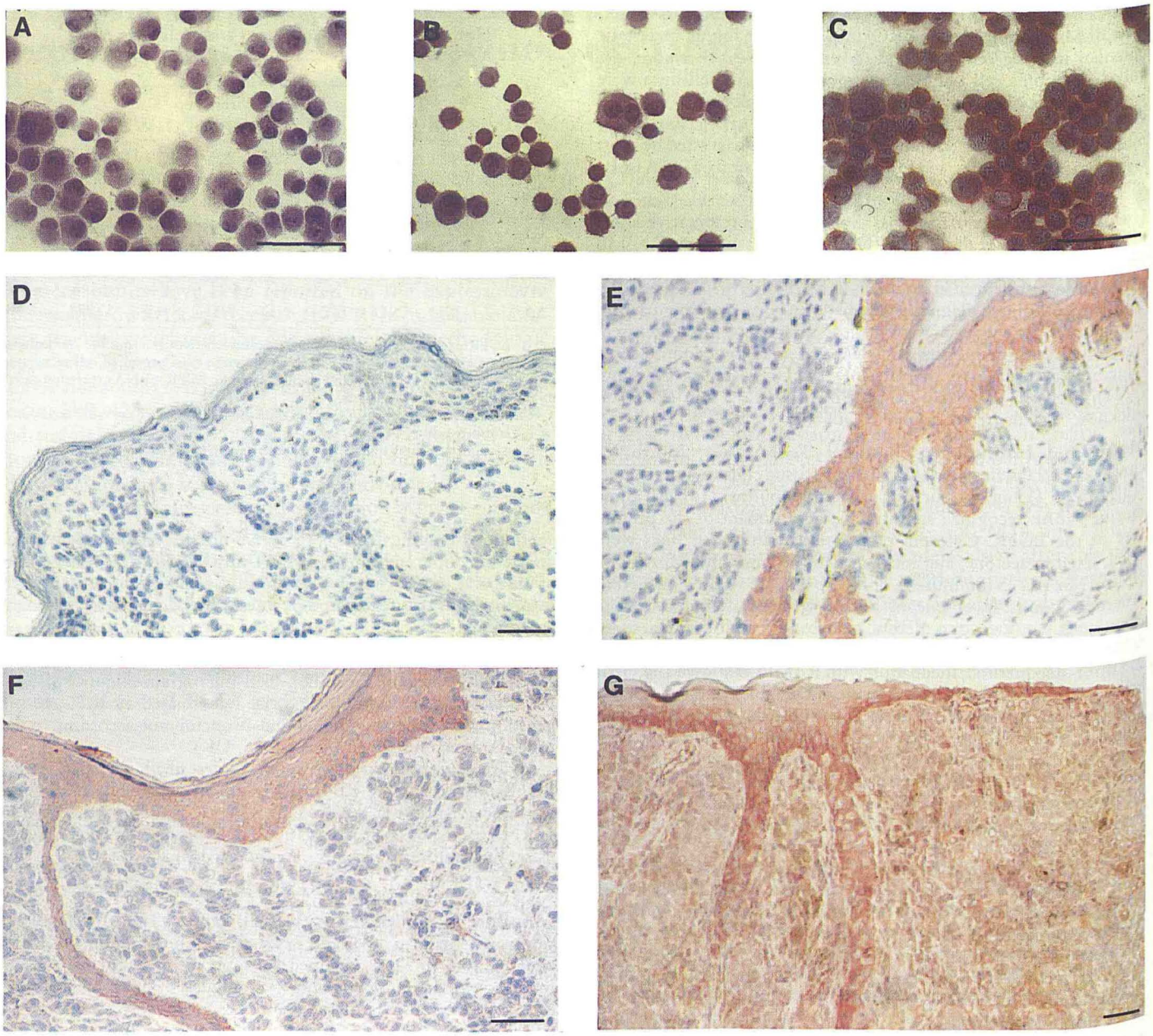

Figure 1. EGFR expression in human melanoma cell lines (*A–C*, cytospin preparations) and human melanocytic lesions (*D–G*, frozen sections). Immunocytochemical staining using the monoclonal antibody 2E9 and a three-step detection method. *A*, cell line IF6 showing no staining, as also found in cell line Mel57; *B*, cell line M14 showing weak staining, as also found in cell line 530; *C*, cell line BLM showing marked staining, as also found in cell line MV3; *D*, common nevocellular nevus; negative control; the primary MoAb 2E9 was omitted; *E*, common nevocellular nevus, nevus cells show no staining, the epidermis shows a marked staining; *F*, common nevocellular nevus, nevus cells show a weak staining in a diffuse pattern, the epidermis shows a marked staining, the mean staining intensity observed in positive dysplastic nevi was similar to the staining shown here; *G*, primary cutaneous melanoma, diffuse intermediate to marked staining of melanoma cells, the mean staining intensity observed in the positive melanoma metastases was similar to the staining shown here. *Scale bar*, 50 μm.

affinity. The quantitative ligand-binding–assay data on EGFR expression in the cell lines correlated well with the semiquantitative immunocytochemical results obtained with the MoAb 2E9 using a three-step detection method. This correlation was not found with the MoAb 425 and the MoAb 225 (Table I).

Northern Blotting of the Cell Lines EGFR gene transcripts (10.5 and 5.8 Kb) were present in four of six human melanoma cell lines tested (Fig 3, table I *(lane C)*]. Cell lines BLM and MV3 showed a high (++), M14 and 530 a low (±), and IF6 and Mel 57 no detectable (−) level of EGFR-mRNA expression. Cell line A431 showed a very high EGFR mRNA expression (not shown). The results on the EGFR expression in the cell lines, as detected with the MoAb 2E9 using the three-step technique, correlated well with the EGFR-mRNA levels in the same cell lines.

Immunohistochemistry of Melanocytic Lesions The expression of the EGFR in frozen sections of the melanocytic lesions as assessed immunohistochemically is summarized in Table 2*A*. For the different categories it shows the percentage of lesions in which ≥5% of the melanocytic cells stained. As in the cell lines M14 and 530 there was a good correlation between the weak immunocyto-

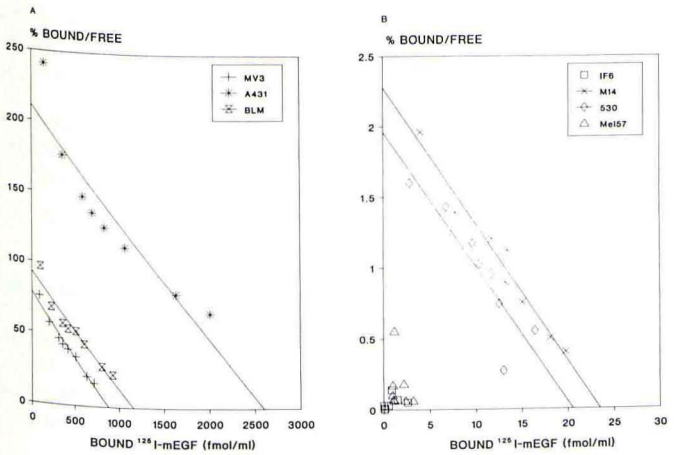

Figure 2. EGFR expression in human melanoma cell lines and cell line A431. Scatchard plot of data obtained with a ligand-binding assay. The ordinate intercept depicts the level of EGFR expression in fmol/ml. Note the scale difference between *A* and *B*.

logic staining, the ligand-binding–assay data, and the mRNA level, weak staining in the sections was regarded as relevant and taken into account.

A clear difference was seen in EGFR expression, when comparing benign and malignant lesions (Table II). The percentage of positive lesions was 19 for common nevocellular nevi, 89 for primary melanomas, and 91 for melanoma metastases. The dysplastic nevi hold an intermediate position (61%). In primary melanomas there was no clear increase in the number of positive lesions with increasing Breslow thickness. Within the group of dysplastic nevi there was no correlation between a history of dysplastic nevus syndrome or the histologic degree of atypia and EGFR expression.

Heterogeneity of EGFR expression was observed in most lesions. In each category of lesions the percentage of positive melanocytic cells per lesion varied (Fig 4). With regard to this parameter there was no correlation with the stage of progression (Table 2*B*). Comparing the different categories, the average staining intensity in positive nevi was weak, in primary melanomas and metastatic melanomas intermediate to marked (Fig 1*D*–*G*).

DISCUSSION

In this study we have compared the level of EGFR expression in six human melanoma cell lines using three MoAb, four immunocytochemical techniques, a multiple-point ligand-binding assay, and Northern blot analysis. The level of EGFR expression in cell line A431, used as a control, was very high, which is in agreement with earlier reports [8,15,29]. A relatively low expression of EGFR could be detected immunocytochemically only when MoAb 2E9 and a three-step staining method were used. Semi-quantitative data obtained with this most sensitive approach correlated well with the quantative data obtained with the ligand-binding assay. Northern blotting results were also in concordance.

We quantified the differences in EGFR expression between melanoma cell lines with a different metastatic behavior in nude mice [16]. Cell lines that frequently give rise to spontaneous lung metastases (BLM and MV3) had a high level of EGFR expression, whereas those with no (IF6 and 530) or only a low (M14 and Mel 57) metastatic potential had no detectable or a low level of expression. These data suggest that an increased level of EGFR expression may be relevant in melanocytic tumor progression. Supporting data, obtained with other cell types, come from studies by Di Fiore et al [12] and Velu et al [30], who found a transformed phenotype of NIH 3T3 cells after induction of EGFR overexpression by transfection experiments and from a report by Wells et al [31] who described a

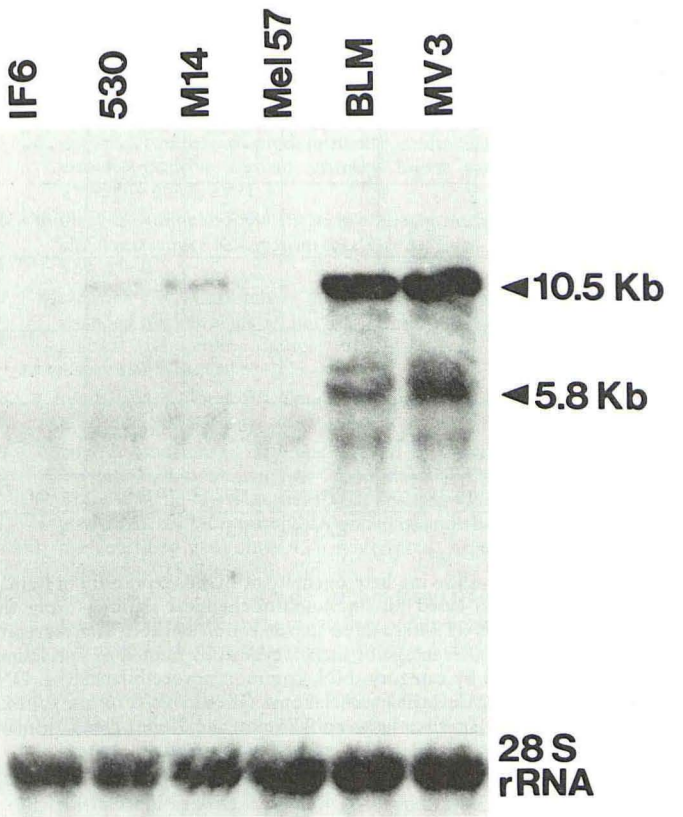

Figure 3. Northern blot analysis of EGFR mRNA expression in human melanoma cell lines using the 0.8-Kb fragment of lambda HER 64 as a probe. Two bands (10.5 and 5.8 Kb) are visible in cell lines BLM and MV3 (intense) and in cell line 530 and M14 (faint). No band could be detected with the cell lines IF6 and Mel 57. As a control on the amount of RNA, the blot was re-hybridized with a 28S-rRNA probe. As molecular weight marker lambda DNA digested with endonuclease Hind III was used.

transformed phenotype of NR6 cells expressing a transfected non-internalizing mutant EGFR.

Because of the good correlation with the ligand-binding–assay results, we considered a weak immunocytochemical staining, as obtained in the cell lines 530 and M14 with MoAb 2E9 and a three-step method, as specific and relevant. This is underscored by the fact that cell lines IF6 and Mel57 were EGFR negative with both techniques.

By doing so, we found differential EGFR expression in benign, premalignant, and malignant human melanocytic lesions. About 20% of common nevocellular nevi, 60% of dysplastic nevi, and 90% of primary and metastatic melanoma lesions showed staining in at

Table II. Expression of EGFR in Human Melanocytic Lesions[a]

	Category[b]					
	NN	DN	PM1	PM2	PM3	MM
A[c]	19	61	87	85	95	91
B[d]	45	67	56	36	42	60

[a] EGFR expression as detected immunohistochemically (MoAb 2E9, three-step method).

[b] NN, common nevocellular nevus; DN, dysplastic nevus; PM1, primary melanoma (Breslow ≤0.76 mm); PM2, primary melanoma (Breslow between 0.76 mm and 3 mm); PM3, primary melanoma (Breslow ≥3 mm); MM, melanoma metastasis.

[c] A, percentage of positive lesions per category.

[d] B, mean of percentages of positive melanocytic cells in positive lesions per category.

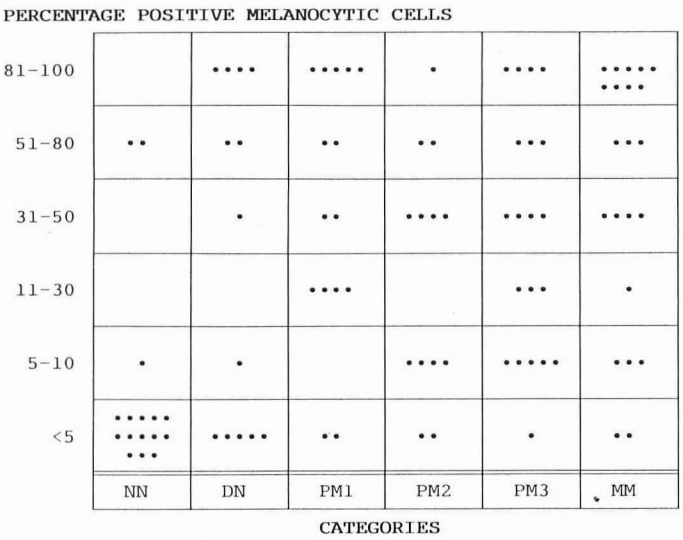

Figure 4. Diagram showing heterogeneity of EGFR expression in human melanocytic lesions, based on immunohistochemical staining using the monoclonal antibody 2E9 and a three-step detection method. Each *dot* represents the estimated percentage of melanocytic cells stained in one lesion. Lesions are ordered by category. NN, common nevocellular nevus. DN, dysplastic nevus. PM1, primary melanoma (Breslow ≤0.76 mm). PM2, primary melanoma (Breslow between 0.76 mm and 3 mm). PM3, primary melanoma (Breslow ≥3 mm). MM, melanoma metastasis.

least 5% of the melanocytic cells. Two previous reports have dealt with EGFR expression in human melanocytic lesions [7,8]. Although Real et al [8] were able to detect EGFR expression in 19 of 36 melanoma cell lines by rosetting-assay analysis, they could not detect EGFR expression in five of five primary melanoma lesions and seven of seven metastases immunohistochemically, using MoAb 225 and an ABC procedure. Elder et al [7] detected EGFR immunohistochemically in two of 11 dysplastic nevi, in three of 16 radial-growth–phase primary melanomas, in eight of nine vertical-growth–phase primary melanomas, and in eight of 10 metastases, using a PAP procedure and MoAb 425. Mature dermal nevi were found negative.

The discrepancy between our results and those of Real et al [8] might be due to the different antibodies used, as equally sensitive immunohistochemical procedures for the detection of EGFR were applied. Our results suggest that the MoAb 225 may have a lower avidity than MoAb 2E9. A factor that may be of major importance is the fact that the two MoAb recognize different epitopes [32]. For the detection of the EGFR expression in melanoma cell lines Real et al used a highly sensitive rosetting assay and found a positive reaction in about half of the lines, which is in agreement with our results. The differences in tissue distribution of EGFR, reported by Elder et al [7] and in the present study, might also be explained by the fact that different MoAb were used. In our comparison of MoAb and detection methods on a panel of melanoma cell lines, we found for the equally sensitive three-step and PAP procedures that the staining signal obtained using MoAb 2E9 was stronger than that using MoAb 425. In addition, the positive correlation between the staining signal obtained with MoAb 2E9, the EGFR level as determined by ligand-binding analysis, and the level of mRNA expressed was not found for MoAb 425. MoAb 2E9 was used as ascites fluid and the MoAb 425 as tissue-culture supernatant. We assume that the concentration and the avidity of the MoAb used have influenced the staining results. Furthermore, the MoAb may recognize different epitopes [13,15]. The outcome of our study of melanocytic lesions was not altered essentially when a 10% margin was applied to score a lesion as "positive." If only the lesions showing a staining intensity of + or more would be regarded relevant, thus excluding the weak-staining signals, no benign and fewer premalignant lesions would be scored positive. However, we were unable to detect differential staining in horizontal-growth–phase primary melanomas as compared to vertical-growth–phase primary melanomas, as reported by Elder et al [7].

From this study we conclude that, although the differences between the various stages of progression are not absolute, we provide further evidence that EGFR expression increases in human melanocytic tumor progression.

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